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Effect of Interstitial Pressure on Epithelial Invasion From Human Mammary Ducts

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14. ABSTRACT
The objectives of this Concept Award are to develop methods to engineer open "ducts" of human mammary epithelial cells in collagen-based gels in vitro, and to study how the distinct pressures in the apical (lumenal) and basal sides of the epithelium affect tumor invasion. This work has yielded a technique to form ducts of immortalized MCF-10A human mammary epithelial cells; application of similar methods to primary cells was unsuccessful in creating stable ducts. Open ducts of MCF-10A cells could be exposed to different apical and basal pressures by interfacing the gels and the open ends of ducts to different liquid reservoirs. This work provides new tools for studying how physical forces affect the progression of human breast tumors in a well-controlled in vitro setting.

15. SUBJECT TERMS
breast cancer, invasion, collagen, interstitial pressure, transmural pressure, in vitro

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INTRODUCTION

The goals of this Concept Award are 1) to engineer a culture model that enables application of distinct apical and basal pressures across human breast epithelium, and 2) to study how changes in these pressures (in particular, the difference in pressure, or "transmural" pressure) affect invasion by transformed cells within the epithelium. Since the growth of breast tumors in humans is often accompanied by an increase in interstitial pressure, it is important to know whether such physical changes promote or retard tumor invasion. Our work is intended to provide in vitro results that address this issue.

BODY

Task 1. Develop methods for organizing cultured human normal and transformed mammary epithelial cells into open ducts that can be exposed to interstitial hypertension (Months 1-4).

1A. Determine culture conditions that enable the normal immortalized human mammary epithelial cell line MCF-10A to adhere and grow in open channels in collagen and Matrigel (Months 1-3).

We have successfully grown MCF-10A cells in 120-micrometer-diameter channels in type I collagen gels and in gels that are doped with up to 20% Matrigel (Figure 1). These cells grew to confluence within three days, thereby forming rudimentary "ducts" that could be perfused. Although the cells initially formed a monolayer, all ducts eventually overgrew to fill the lumen at discrete locations. We did not discern any pattern to this overgrowth (e.g., no regular spacing or timing), which typically required 1-2 weeks to manifest. Addition of retinoic acid (1 μM), which has been claimed to promote the formation of hollow acini in other culture systems (Montesano and Soulié, 2002), did not retard the overgrowth of ducts. To date, we can form MCF-10A ducts that remain open from days 3-7 post-seeding.

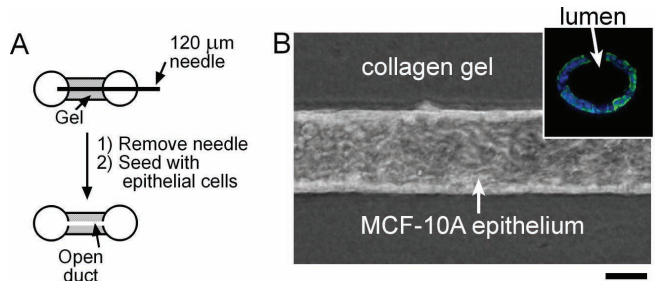

Figure 1. A) Method to form open human mammary epithelial ducts. B) Phase-contrast image of a thick-walled duct of MCF-10A cells. *Inset*, cross-section of duct, stained for cytokeratin (green) and DNA (blue). Slight deformations result from sample preparation. Scale bar refers to 50 μm.

1B. Determine culture conditions that enable normal primary human mammary epithelial cells (HMECs) to form open channels in collagen and Matrigel (Months 2-4).

We have had much less success with forming open ducts of HMECs in collagen gels. HMECs initially adhered and spread well in the gels, but strangely, they invariably began to adopt fibroblastic morphologies over the span of 3-4 days. Thus, HMECs did not form monolayers within the channels, instead organizing into clusters of spindly cells. In consultation with Prof. Celeste Nelson (Princeton) and Martha Stampfer (LBNL), we suspected that the cells may undergo an epithelial-mesenchymal transition within the channels, or that the cells are somehow no longer epithelial due to in vitro culture drift. We tried forming HMEC ducts in the presence of the antioxidant N-acetylcysteine (5 mM) or the broad-spectrum matrix metalloproteinase (MMP) inhibitor GM6001 (10 μM), since it has been reported that reactive oxygen species or MMPs can promote the acquisition of mesenchymal phenotype (Radisky et al., 2005); unfortunately, we found that these compounds did not preserve the epithelial phenotype of HMECs in collagen channels. Culture with dibutyryl cyclic AMP (400 μM), phosphodiesterase inhibitor Ro-20-1724 (20 μM), Src kinase inhibitor PP1

(1 μM), or NFκB inhibitor (1 μM), or supplementation of gels with up to 35% Matrigel, also did not prevent the shift to spindly morphology. Oddly, HMECs formed epithelial monolayers on flat collagen gels, and did not adopt fibroblastic morphologies on flat gels.

1C. Immunostain sections of MCF-10A or HMEC ducts for keratins, E-cadherin, and vimentin to characterize the structural organization of these ducts (Months 1-4).

We have stained MCF-10A ducts for cytokeratins using agarose-embedded sections (Figure 1B, inset). These ducts appeared to form a layer with a thickness of 1-2 cells. MCF-10A cells expressed keratins non-uniformly in ducts. These results are consistent with those observed in MCF-10A layers on porous filters (Marshall et al., 2009; Stull et al., 2007).

1D. Introduce pre-labeled, transformed MDA-MB-231 cells into already-formed MCF-10A or HMEC ducts. Confirm the presence of the transformed cells by fluorescence microscopy (Months 2-4).

Not done. We felt this task was premature until we could stably control transmural pressures in ducts (Task 1E).

1E. Construct channels in collagen and Matrigel so that the interstitial pressure of a gel can be set independently from the pressure in the lumen of a channel. Determine the mechanical stability of channels exposed to a pressure of 20 cm H_2O over one week (Months 1-3).

We have successfully constructed open MCF-10A ducts in which the apical (lumenal) and basal compartments were exposed to distinct hydrostatic pressures. To accomplish this task, we designed a new culture setup that provided fluidic contact between the gel and "drains" (Figure 2). Thus, the two open ends of a duct and the drains adjacent to a gel could be subject to different pressures. As in Tasks 1A and 1B, we found that MCF-10A ducts with drained gels eventually filled in parts of the lumens, while HMECs did not form ducts in this setup.

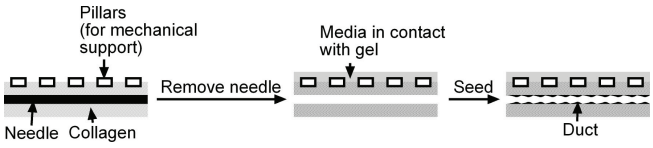

Figure 2. Method to form open human mammary epithelial ducts that can be exposed to changes in interstitial pressure. MCF-10A ducts that were formed in this setup looked essentially identical to those without a drained basal compartment.

1F. Use the conditions identified in (1A) and (1B) and the channels formed in (1E) to create mammary epithelial ducts that can be exposed to interstitial pressures of up to 20 cm H_2O (Months 3-4).

Using the setup invented in Task 1E, we could routinely expose ducts to positive interstitial pressures, as long as these pressures were matched by the pressures in the duct lumen. We have been unable to obtain negative transmural pressures (i.e., where interstitial pressure exceeds lumenal pressure) without delamination of epithelium from the gel.

Task 2. Determine the effect of elevated interstitial pressure on invasion from ducts of mammary epithelial cells (Months 5-12).

Until we can stably apply negative transmural pressures (i.e, without ductal collapse), we felt it was premature to begin working on Tasks 2A-2E. Given our experience with cellular delamination in other systems (Price et al., 2010; Wong et al., 2010), we suspect that the contractility of the duct may amplify the effect of a negative transmural pressure and thus enhance the tendency of epithelium to detach from the gel. To counteract this tendency, we

plan to try applying transmural pressures across flat layers of MCF-10A cells supported on a gel.

2A. Vary the interstitial pressure from 0 to 20 cm H_2O in steps of 5 cm H_2O, while holding lumenal pressure at 0 cm H_2O. Vary the fraction of epithelium that is MDA-MB-231-derived from 0% to 20%, in steps of 5%. At each pressure and ductal composition, obtain daily counts of the total number of invasive cells by phase-contrast microscopy. Obtain daily counts of the number of invasive MDA-MB-231 cells by fluorescence microscopy (Months 5-8).

2B. Expose ducts to interstitial pressure of 20 cm H_2O for 1-7 days, and then revert the pressure back to 0 cm H_2O. Determine the extent of invasion for a total time in culture of 7 days. Determine that minimum time of exposure required to induce irreversible invasion by 7 days in culture (Months 6-9).

2C. Vary the lumenal pressure from 0 to 20 cm H_2O, while holding interstitial pressure at 10 cm H_2O, and obtain daily counts of total number of invasive cells and number of invasive MDA-MB-231 cells. From these data and the data from (2A), determine if interstitial pressure or transmural pressure correlates better with the effect on invasion (Months 9-12).

2D. Immunostain ducts that have been exposed to set interstitial and lumenal pressures for one week for E-cadherin, keratin, and vimentin. Determine if interstitial pressure or transmural pressure correlates best with changes in staining intensity (Months 5-12).

2E. Analyze the global gene expression profile of ducts exposed to interstitial pressure of 0 or 20 cm H_2O. We have chosen only two pressures for these experiments because microarray assays are expensive and many ducts will be required at each pressure to obtain sufficient mRNA for hybridization (Months 7-12).

KEY RESEARCH ACCOMPLISHMENTS

- Development of a method to form open MCF-10A ducts in collagen gels
- Development of a method to form open MCF-10A ducts in which basal and lumenal pressures can be independently controlled

REPORTABLE OUTCOMES

- This work has provided preliminary data for a proposal to the FY2010 DoD Breast Cancer Idea Award competition, entitled "Role of Elevated Interstitial Pressure on Dysfunction of Mammary Tumor Epithelium and Stroma".
- A manuscript on this work is in early stages of preparation.

CONCLUSION

This work has developed new techniques for forming open ducts of human breast epithelium and for applying pressures to them. These methods will enable researchers to better mimic the physical environment of human breast tumors in vitro. Recent studies have suggested that physical signals play an important role in breast tumor progression (Levental et al., 2009; Paszek et al., 2005), although the effect of elevated interstitial pressures is unclear. Our results enable the stable application of positive, but not negative, transmural pressures to engineered mammary ducts. Further work to subject epithelium to negative transmural pressures without delamination (by forming the epithelium on a flat gel rather than in a channel) will broaden the usefulness of these methods in the study of human breast cancer progression.

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APPENDICES

None.

SUPPORTING DATA

Figures and data are presented in the body of the text.